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September 08, 2004

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APPLICATION NUMBER: *60/509,546*
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
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Additional inventors are being named on the second *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Method for freezing and thawing of viable cartilage

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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OR

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 18 ☐ CD(s), Number

☑ Drawing(s) *Number of Sheets* 1 ☑ Other (specify) Transmittal,postcard

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.

☑ A check or money order is enclosed to cover the filing fees.

☑ The Director is herby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 14-0112

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE Amount ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE

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Date October 9, 2003

REGISTRATION NO. 40,669 *(if appropriate)*

Docket Number: 25761

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PROVISIONAL APPLICATION COVER SHEET
Additional Pag

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Docket Number 25761

INVENTOR(S)/APPLICANT(S)

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Number 2 of 2

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MAIL STOP PROVISIONAL PATENT APPLICATION
Attorney Docket: 25761

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Udi DAMARI et al.

Serial Number: NOT YET ASSIGNED

Filed: October 9, 2003

Title: **METHOD FOR FREEZING AND THAWING OF VIABLE CARTILAGE**

TRANSMITTAL LETTER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450
PPP
Commissioner:

Submitted herewith for filing in the U.S. Patent and Trademark Office are the following **PROVISIONAL APPLICATION**:

1) Transmittal Letter
2) Provisional Application Cover Sheet
3) 19 page Provisional Application consisting of:
   15 pages Textual Specification
   3 pages of 17 claims
   0 abstract of the disclosure
   1 sheet drawing
4) Check No. 1956 $ 80.00 for filing fee as a small entity
5) Postcard for early notification of serial number

The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-0112

Respectfully submitted,
NATH & ASSOCIATES PLLC

By: _____
Todd L. Juneau
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Date: October 9, 2003
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METHOD FOR FREEZING AND THAWING OF VIABLE CARTILAGE

FIELD OF THE INVENTION

The present invention relates to freezing and thawing viable cartilage and methods for freezing and thawing such tissue.

LIST OF REFERENCES

The following references are brought to facilitate description of the background of the present invention, and should not be construed as limiting the patentability of the invention:

1. Muldrew, K. et al., Cryobiology 43, 260-267 (2001);
2. Higgs, G.B. and Boland, A.L, Proceedings of the International cartilage Repair Society's Second Symphoium, November 1998;
3. McGoveran, B.M. et al., The Journal of Knee Surgery, vol.15, No.2 Spring 2002;
4. US patent 5,131,850 to Kelvin G. M.;
5. US patent 6,488,033 to Cerundolo D. G;
6. US patent 5,873,254 to Arav A.;
7. US patent application 20030064357 to Arav A.;
8. PCT/IL03/00026 to Arav A.

The above publications will be referenced below by indicating their number from the above list.

BACKGROUND OF THE INVENTION

Adult cartilage is a connective tissue populated by chondrocytes embedded in a dense extra cellular matrix (ECM) composed of a collagenous fiber network. Functional articular cartilage is critical to proper joint function. Unfortunately articular cartilage has low self-repair ability and therefore defects are prone to cause abnormal joint biomechanics, leading in the long run to degenerative changes.

Treatments strive for a pain-free range of motion in the joint that provides enduring function, which enable a young patient to participate in a wide range of activities [3]. The list of operative treatment options for hyaline cartilage injury currently utilized by orthopaedic surgeons include microfracture, chondral abraisonplasty, osteochondral autograft transplantation (OATS or mosaicplasty), autologous chodrocyte transplantation (ACI), fresh osteochondral allografts, autologous perichondral or periosteal transplantation, and transplantation of bioabsorbable or non-bioabsorbable matrices. The ultimate alternative is, of course, partial or total replacement of the joint with prosthesis [2]. Nevertheless the management of large full thickness osteochondral lesions remains an enigma.

Currently, the method of choice to repair cartilage damage that is greater than 3 cm in diameter and 1 cm in depth is the implantation of tissue [3]. Normally, the implanted tissue (comprising bone and cartilage) is taken from a cadaver, from a site that is most similar to the organ that is in need of repair in the recipient. This allows the implanted tissue to have the most similar shape, arrangement (e.g. of bone and cartilage tissue) and weight bearing characteristics as the tissue of the implant site. This implant (or graft) is often named "*allograft*" since the graft is taken from one individual and implanted in another.

Currently, cartilage transplantation is limited to fresh grafts. This is mainly due to the fact that the post-implantation viability of the chondrocytes is necessary for the long-term maintenance of the biomechanical properties of the cartilage graft. Chondrocytes in cartilage are enclosed in lacunas within the ECM, such that if one

a cell dies within a lacuna it cannot be replaced by a cell migrating thereto. Thus, unlike bone grafts that may comprise dead bone tissue (which will be later populated by bone cells that would migrate into the implant) the cartilage graft must provide viable cartilage cells.

However, cartilage cells can be maintained viable within cartilage for a restricted period of time and must therefore be transplanted within a few days (no more than 30) from the moment of donation [4]. This does not normally allow sufficient time to test the donated tissue for undesired agents or traits such as transmittable diseases. It also reduces the chances of finding the best donor-recipient match.

Long-term banking is the only strategy that can ensure an adequate and safe supply of cartilage and the need for matching tissue type and site-specific requirements (size, cartilage thickness etc.).

Isolated chondrocytes (i.e. not within cartilage ECM) have been successfully cryopreserved, [1]but so far cartilage cryopreservation and control rates of freezing and thawing did not achieve an acceptable degree of cartilage viability [3].

US Patent No. 5,131,850 [4] purported to disclose in 1992 a method for cryopreserving musculoskeletal tissues such as cartilage. However, it is commonly accepted that this cannot be achieved and the same assignee clearly accepts (in a later patent) that "*Osteoarticular allografts, however, have not typically been used because osteoarticular cells do not survive the freezing or cryopreservation process*" [5].

In the work done by Muldrew et al. [3] cartilage was cryopreserved by exposure of the cartilage to step-wise decreasing external temperature. This method resulted in improved chodrocytes recovery within the thawed cartilage. Nevertheless there was considerable variability in the cells' survival rates within the experimental group and the mean cell recovery was not appreciably improved.

An advanced technology for freezing is the "Multi-temperature gradient" (MTG) directional solidification, which is based on the invention disclosed in US 5,873,254. In this technology, the sample is moved at a constant velocity (V) through temperature gradients (G) so the cooling rate (G x V), ice front propagation are controlled and the velocity of the movement of the sample determines the morphology of the ice crystals formed within the sample. This method also enables the incorporation of controlled seeding into the freezing process.. This method was utilized for example for freezing various biological samples in containers of various dimensions (e.g. sperm, blood, oocytes 6, 7) and even whole organs [8].

Glossary

The term "*viable cartilage*" in the context of this invention means any tissue comprising viable cartilage cells (chondrocytes) embedded in cartilage extra cellular matrix (ECM), whether or not comprising other elements including cells of other types and/or ECM. Such cartilage may be taken from any source, including, for example, hyaline cartilage (such as the cartilage present in the tip of joints) and fibrocartilage (such as the cartilage present in the ears and in the inner parts of the nose). Non limiting examples for non-cartilage cells and tissues that may be included in a viable cartilage sample are cells and/or extra cellular matrix of bone, tendon, ligament, etc. In order for the cartilage to be deemed viable, at least some of the chondrocytes embedded therein must be viable, preferably 30% or more, 50% or more, 65% or more or even 69% or more.

The term "*viable [cells/tissue]*" in the context of this invention means cells or tissue (as the context requires) comprising cells that are capable of surviving provided that they are given the necessary conditions (e.g. nutrients, temperature and the like). When applied to frozen cells/tissue, the term "*viable*" denotes such cells or tissues that are capable of remaining viable after being thawed.

The term "*frozen*" in the context of this invention means having a temperature in which all biological processes are practically ceased.

The term "*freezing temperature*" in the context of this invention means a temperature below which a liquid to crystal phase transition occurs.

The term "*melting temperature*" in the context of this invention means a temperature above which there is no more recrystalization of ice but only transition from ice to liquid.

The term "*glass transition temperature*" (Tg) in the context of this invention means a temperature which below it a system is increased in its viscousity until it is no longer liquid but is turned into an amorphous solid in a stable thermodynamic state.

The above temperatures are different for each solution, and are a product of the solution's composition. The temperatures can be determined, at least approximately, by methods known in the art. In the context of this invention, a temperature is deemed to be "*about*" or "*substantially equal*" to a given temperature (e.g. to a melting temperature) when the difference in temperature is no greater than 10°C, more preferably no more than 5°C, 1°C or even 0.5°C, and most preferably no more than 0.01°C.

The term "*container*" in the context of this invention means any sort of container capable of containing viable cartilage and capable of withstanding the freezing and/or thawing of the invention without damage, such that the viable cartilage may be protected from spillage and/or contamination. Non-limiting examples of such containers are tubes, bags, straws, sacs and laboratory dishes.

SUMMARY OF THE INVENTION

The method of the present invention is aimed at freezing and thawing of viable cartilage.

One aspect of the present invention is a method for the generation of frozen viable cartilage using a cryopreservation solution, said method comprising:

(a) providing a container containing viable cartilage in said cryopreservation solution at a temperature above the freezing temperature of the cryopreservation solution;

(b) moving said container along one or more consecutive temperature gradients ranging from a temperature above said freezing temperature to a temperature below said freezing temperature such that the sample would be cooled at a cooling rate of 0.01°C/min to 3°C/min, such that the frozen viable cartilage is obtained.

The cooling rate of above Step (b) is a result of the temperature gradients along which the container is moved (°C/mm) and the velocity of movement along the gradient (mm/min). The velocity of movement of the method of the invention may be any velocity that would achieve the desired cooling rate, and preferably between 0.0001 mm/sec and 5 mm/sec. The temperature gradient along which the sample is moved may be any gradient that would achieve the desired cooling rate, and preferably between 0.1°C/mm to 50°C/mm.

According to yet another aspect of the present invention, a method is provided for thawing frozen viable cartilage comprising a cryopreservation solution, to obtain thawed viable cartilage, said method comprising:

(i) providing a container containing frozen viable cartilage at an initial temperature below the glass transition temperature of the cryopreservation solution;

(ii) warming said viable cartilage from said initial temperature to an intermediate temperature being at least about said glass transition temperature or above said glass transition temperature but no more than

the temperature wherein recrystalization begins to occur at a rate sufficiently slow to minimize fracture of said viable cartilage;

(iii) warming said viable cartilage from said intermediate temperature to a temperature that is at least substantially equal to the melting temperature of the cryopreservation solution at a rate sufficiently high to minimize recrystalization, such that thawed viable cartilage is obtained.

The present invention is not limited to a specific method of changing the temperature of the viable cartilage, and this may be done in any manner that would achieve the desired rate of temperature change. Thus at any step of the method of this invention the viable cartilage may be warmed for example by immersion of the container in a fluid having a warmer temperature than the container or by directional movement of the container along one or more temperature gradients.

A preferred rate of thawing in above step (ii) is between 0.1°C/min and 200°C/min, and more preferably 90°C/min. Likewise, a preferred rate of thawing in above step (iii) is between 100°C/min and 1000°C/min, and more preferably 200°C/min.;

As detailed above, the present invention provides frozen viable cartilage. Such cartilage may remain viable for an extended period of time (practically – indefinitely), and may thus be banked. This would allow all useful or necessary tests to be performed before the tissue is transplanted (e.g. screening for infectious diseases). Banking may enable doctors to choose a graft from a larger reservoir of tissue and thus allow better donor-recipient matching (e.g. size, source and target site, shape).

BRIEF DESCRIPTION OF THE DRAWINGS

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

Fig. 1 is a graphical representation of the temperatures measured during freezing within a bone portion and a cartilage portion of an osteochondral graft while being frozen in accordance with this invention;

DETAILED DESCRIPTION OF THE INVENTION

In the following, all materials were purchased from Sigma St. Louis, USA unless specified otherwise.

1- freezing of osteochondral cylinders from sheep.

Fresh cadaver sheep legs were purchased from a slaughter house (Holon Slaughter house, Israel), and all manipulations of tissue samples were done in a sterile manner. Osteochondral cylinders, 12 mm in diameter, were drilled from sheep knee chondyle using a power surgery drill (Imex, Veterinary Inc. Texas, USA). Harvested cylinders were maintained in a buffered physiological solution containing 0.9% NaCl (Sigma, St. Louis, USA) and 3% antibiotics (Penicilin/Streptomycin/ Nystatin, Biological Industries, Beit Haemek, Israel) until completion of harvesting.

10 ml cryopreservation solution (comprising nutrient mixture F-12 (HAM), 1.78M Ethylene Glycol and 1% antibiotic (penicilin/streptomycin/nystatin)) was put in a conventional 16 mm (in diameter) glass tube. The harvested cylinders were inserted into the tubes with the cartilage part of the cylinder close to the tip of the tube. Cylinders were held at 4°C for a period of 30 minutes to 6 hours before freezing.

The cylinders were frozen using Multi Thermal Gradient (MTG) freezing apparatus (IMT, Israel) comprising 4 cooling blocks. The cylinders were inserted into a first block and then moved through the blocks at 0.022mm/sec. The block temperatures, at their distal ends, were 0°C, -6°C, -46°C and -86°C. The cooling rates were 0.1°C/min from 0°C to -6°C and 0.4°C/min from -6°C to -86°C. After freezing the cylinders were transferred for storage in liquid nitrogen (LN) (temperature -130°C- -196°C).

Fig. 1 is a graph which depicts the temperature change within the cylinder during freezing, measured using two thermocouples (TCs) (ALMEMO^{R} 2290-4). One TC was inserted into bone tissue within the cylinder and the other TC was inserted within a cartilage portion of the cylinder. As seen, the cylinder was frozen in two different rates, the first rate being slower than the second rate. The cartilage portion that was placed near the leading end of the tube froze slightly faster than the bone portion. Between the two freezing rates there is a short isotherm, wherein the temperature is measured to rise rather than fall. This is due to the fact that crystallization, which occurs at the freezing point, is an exogenous reaction. In directional freezing, the heat so generated is transferred to the warmer portion of the sample (i.e. against the direction of the temperature gradient). Thus the rising of temperature does not substantially affect the arte of freezing of the cylinder.

2- Thawing of osteochondral cylinders from sheep.

Tubes containing frozen articular cartilage cylinders (frozen according to the freezing protocol detailed in point 1 above) were removed from LN and were held at room temperature for 100 seconds (slow warming). They were then transferred to a 68°C water bath for 10 seconds and then to a 37°C water bath until cylinders were brought back to physiological temperatures (rapid warming).

The cryopreservation solution was washed by transferring the thawed cylinders through a series of solutions with decreasing concentrations of sucrose

(0.5M, 0.25M and 0.125M). Sucrose (Sigma, St. Louis, USA) was dissolved in a solution composed of 89% F-12 HAM medium, 1% antibiotic (Penicilin/Streptomycin/Nystatin) and 10% FCS (all from Biological Industries Beit Haemek, Israel). After at least 5 minutes in each solution the thawed cylinders were kept in a F12 solution with 1% antibiotics Penicilin/Streptomycin/Nystatin-Bilological Industries Beit Hamemek, Israel) and 10% FCS (Biological Industries Ltd.).

3- Survival of chondrocyes.

Different *in vitro* assays were used to assess the quality and viability of chondrocytes populating articular cartilage cylinders after freezing and thawing as described in points 1 and 2 above. The results of thawed cartilage cylinders were compared to those of fresh ones.

Cartilage cultures

Thawed cartilage cylinders were obtained in accordance with point 2 above . Fresh cartilage cylinders were harvested along with the cylinders that were frozen but were assayed as follows, upon harvesting and without freezing. The cylinders were cut to cubes measuring ca. 2x2x1 mm and were assayed as follows.

Cell viability assays

Table 1 summarizes the experimental results of experiments conducted to show the post-thaw viability of cartilage cells after being frozen and thawed in accordance with this invention. Sample 1 was fresh cartilage. Samples 2-4 were frozen at the following respective velocities of movement and cooling rates: Sample 2: 0.1mm/sec, 0.6°C/min; Sample 3 = 0.05mm/sec, 0.6°C/min; Sample 4 = 0.025mm/sec, 0.4°C/min.

Table 1. *In vitro* sheep knee cartilage freezing results

Sample				
Cell culture*	100%	40%	40%	80%
XTT* OD/mg tissue	100%	5%	44%	40%
LIVE/DEAD Viability (average ±sd); N=5	63.46±11/69	32.48±3.06	41.52±4.24	44.86±6.48
Survival*	100%	50%	65%	69%

* the percentage was calculated in comparison to fresh cartilage (sample 1)

The thawed or fresh tissue cubes (5 samples of each) were cultured in F12 (Biological Industries Ltd., Beit Haemek, Israel) supplemented with 10% FCS (Fetal Calf Serum Biological Industries Ltd., Beit Haemek, Israel).

The experimental protocols corresponding to Table 1 were as follows:

1. Cell culture assay

The samples were observed during incubation to confirm that chondrocytes migrated out of the cubes. Table one shows the proportion of cultures in which cartilage cells migrated out of the cubes within two weeks.

1. XTT-cell metabolism assay

The XTT reagent kit (Biological Industries Ltd., Beit Haemek, Israel) was used in accordance with the manufacturer's instructions. In this method the optical density (OD) is proportional to the number of living cells and their metabolic state.

Cartilage slices were incubated in a 96 well micro titer plates in complete culture medium (89% F12, 1% antibiotics, 10% FCS). A well containing slices of cartilage boiled for 2 minutes was used as control for non specific uptake by dead cells, and showed no significant difference in OD from the background. OD results

were divided in cartilage wet weight to normalize for small differences in sample size.

Cell viability assay

The LIVE/DEAD Viability Kit (L-7011 Molecular Probes, Oregon, USA), was used in accordance with the manufacturer's instructions. In this method live cells with intact membranes are observed as bright green, whereas cells with damaged membranes are observed red. Viability is expressed as number of green cell/total number of cells (green + red cells).

Harvesting and handling of Cartilage

In the above example, cartilage was obtained from sheep. However, the present invention may be carried out also in respect of tissue harvested from any other animal, including humans. After freezing and/or thawing the sample may be used for any purpose, including implantation in the donor or in a different recipient, whether or not of the same species, or for any other purpose such as research. The cartilage may be of any shape or size and can be harvested in any desired manner. However, to avoid contamination cartilage that is intended for implantation must be handled in a sterile manner.

Preferably, the frozen tissue is an osteochondral graft comprising articular cartilage removed from the femoral chondyls of the knee and may be in the form of a cylinder measuring 3mm to 50mm in diameter.

The harvested cartilage may be maintained for the duration of the harvesting procedure and for a short time prior to freezing under any conditions compatible with the tissue survival, and preferably in 0.9% NaCl solution containing 3% antibiotic solution that is microbially effective, while sparing the cells which are important for long-term tissue maintenance. The tissue is normally kept in room

temperature until insertion into a cryoprotecting solution and then moved to 4°C, where it may be maintained for several hours before freezing.

Cryopreservation

Any conventional buffered physiological solution can be used in practicing the present invention. For example - tissue culture media and simple buffered salt solutions may be used. A cryopreserving agent is added in solution to the osteochondral tissue to protect the cells during freezing, preferably ethylene glycol, although other suitable cell-penetrating organic solutes can be used, such as DMSO, polyalcohols (for example, ethylene Glycol propylene glycol, glycerol and butane diol); and alkyl sulphoxides (for example, methyl ethyl sulphoxide, diethylsulphoxide, dibutylsulphoxide, methylbutyl sulphoxide, and ethylbutylsulphoxide).

The cryopreservation solution may comprise for example 5 to 200ml of buffered physiological solution and a cell-penetrating organic solute in a concentration from about 0.5M to about 3M. Antibiotic solution of any kind may be included having the preferred concentration of 0.5% to 5%. The volume of solution used is such that the tissue would be completely immersed therein and can be easily determined by one skilled in the art, and is dependent upon the size of the tissue to be preserved.

Freezing can be done using any apparatus or method that will allow directional freezing of the cartilage, such as the Multi Thermal Gradient (MTG) freezing apparatus (IMT, Israel) that was used above. Block temperatures should impose on the viable cartilage a gradient beginning at a temperature above the freezing temperature of the solution, preferably between 5°C and 0°C, and ending at a temperature below the freezing temperature wherein recryslaization is practically non-existent. The cartilage may be cooled at any cooling rate that is

sufficiently slow to prevent damage to the chondrocytes and preferably between 0.01°C/min and 3°C/min.

In addition, it is known in the art to be advantageous to initiate controlled seeding in the sample, to avoid uncontrolled ice nucleation, and thus to minimize the damage to the tissue from the ice propagation. This can be achieved in any manner including for example touching the leading end of the tube with a very cold object (e.g. a small amount of liquid nitrogen) before starting the freezing procedure.

After freezing is completed the tubes may be stored in any cold storage facility at -130°C to -196°C. The samples may be kept practically indefinitely, and in any case may survive storage from 24 hours to 6 months before thawing.

Thawing

It is appreciated by a person skilled in the art of the invention that according to the thawing method of the invention the viable cartilage is thawed in two stages. In the first stage the tissue was brought from the storage temperature to a glass transition state by bringing it to a temperature between -130°C and -30°C. This stage is conducted at a slow warming rate (0.1°C/min-200°C/min) in order to avoid fracture of the tissue. In the second stage samples are brought to the melting point by bringing their temperature to 0°C at rapid warming rate (100°C/min-1000°C/min) in order to avoid recrystalization which is known to damage biological entities.

After thawing, the cryoprotectant is removed as follows: The viable cartilage thawed as described above is removed using a sterile technique and placed in a solution containing biocompatible sugar, a serum of any species and antibiotics in a buffered physiological solution for 5 to 10 minutes. Any non-cell membrane permeable biocompatible sugar, polyol or other organic solute can be used, such as

sucrose, mannitol, sorbitol, trehalose, fructose, glucose, raffinose, maltose, xylitol, amino acids or the like.

The dilution of the cryoprotectant concentration by the biocompatible sugar solution is preferably in decreasing steps of at least half the molarity of the previous step. Thus, if the original cryoprotectant concentration is 2M, the first dilution step would employ 1M sugar.

The above examples are by no way limiting, and the methods of the invention may be carried out in many different variations, some of which are detailed below:

CLAIMS:

1. A method for the generation of frozen viable cartilage using a cryopreservation solution, said method comprising:

(a) providing a container containing viable cartilage in said cryopreservation solution at a temperature above the freezing temperature of the cryopreservation solution;

(b) moving said container along one or more consecutive temperature gradients ranging from a temperature above said freezing temperature to a temperature below said freezing temperature such that the sample would be cooled at a cooling rate of 0.01 °C/min to 3°C/min, such that the frozen viable cartilage is obtained.

2. The method of Claim 1 wherein the the velocity of movement along the temperature gradient in step (b) is between 0.0001 mm/sec and 5 mm/sec.

3. The method according to Claim 1 or 2, wherein the temperature gradients in step (b) are between 0.1°C/mm to 50°C/mm

4. The method according to anyone of Claims 1 to 3 wherein step (b) further comprises controlled initiation of seeding of freezing

5. The method of Claim 4, wherein the seeding is initiated by touching the leading end of the container with a cold object;

6. The method of Claim 5, wherein the cold object is liquid nitrogen.

7. The method according to anyone of Claims 1 to 4, further comprising after step (b):

(c) transferring the container to storage at a temperature below the freezing point

8. The method according to Claim 7, wherein said storage is in liquid nitrogen (LN).

9. Frozen Cartilage obtainable by performing the steps of anyone of Claims 1 to 8.

10. A method for thawing frozen viable cartilage comprising a cryopreservation solution to obtain thawed viable cartilage, said method comprising:

(a) providing a container containing frozen viable cartilage at an initial temperature below the glass transition temperature of the cryopreservation solution;

(b) warming said viable cartilage from said initial temperature to an intermediate temperature being at least about said glass transition temperature or above said glass transition temperature but no more than the temperature wherein recrystalization begins to occur at a rate sufficiently slow to minimize fracture of said viable cartilage;

(c) warming said viable cartilage from said intermediate temperature to a temperature that is at least substantially equal to the melting temperature of the cryopreservation solution at a rate sufficiently high to minimize recrystalization, such that thawed viable cartilage is obtained.

11. The method according to Claim 10, wherein the temperature change rate at Step (b) is between 0.1°C/min and 200°C/min;

12. The method according to Claim 11, wherein the temperature change rate at Step (b) is90°C/min.

13. The method according to anyone of Claims 10 to 12, wherein the temperature change rate at Step (c) is between 100°C/min and 1000°C/min;

14. The method according to Claim 13, wherein the temperature change rate at Step (c) is 200°C/min.

15. The method according to any one of Claims 10 to 14 wherein anyone of steps (b) or (c) is achieved by moving the container along one or more temperature gradients.

16. Thawed Cartilage obtainable by performing the steps of anyone of Claims 10 to 15;

17. Thawed Cartilage obtainable by:

(a) performing the steps of anyone of Claims 1 to 8; and

(b) performing the steps of anyone of Claims 10 to 15;

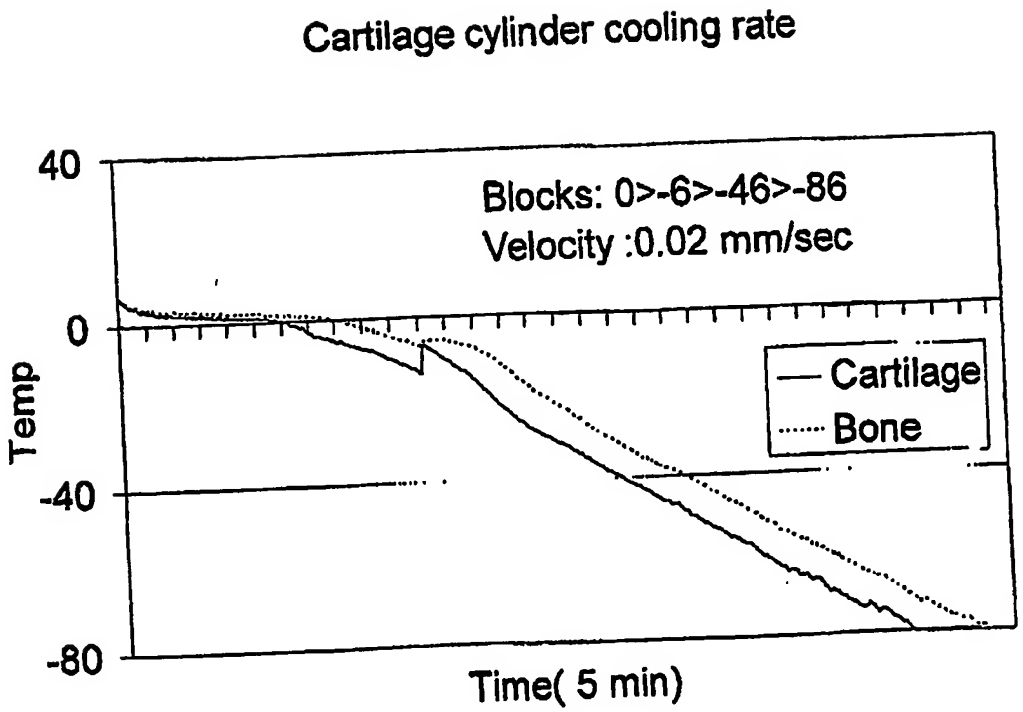
Cartilage cylinder cooling rate
40
0
-40
-80
Temp
Blocks: 0>-6>-46>-86
Velocity :0.02 mm/sec
Cartilage
Bone
Time(5 min)

FIG. 1